Reference: *Biol. Bull.* **199:** 59–67. (August 2000)

Fertilization in *Callochiton castaneus* (Mollusca)

JOHN BUCKLAND-NICKS[1,*] AND ALAN N. HODGSON[2]

[1]*St. Francis Xavier University, Department of Biology, Antigonish, Nova Scotia, Canada B2G 2W5; and*
[2]*Rhodes University, Department of Zoology and Entomology, Grahamstown 6140, South Africa*

Abstract. A fine-structural study of fertilization in *Callochiton castaneus* has revealed that the mechanism of sperm penetration into the egg is intermediate between the primitive condition found in members of the order Lepidopleurida and the more derived condition found in the Chitonida. *C. castaneus* sperm have the long needlelike nuclear filament and reduced acrosome that characterizes all Chitonida, but they have retained several plesiomorphic features such as an unspecialized mid-piece and a lack of flagellar reinforcement. As in some Lepidopleurida but unlike any Chitonida, the egg hull in this species comprises a thick, smooth jelly coat permeated by pores that permit sperm rapid access to the vitelline layer. The jelly coat is delicate and quickly dissolves when a sperm concentrate is used, suggesting that excess acrosomal enzymes may be responsible. Once the sperm have penetrated the vitelline layer, the long nuclear filament bridges the gap to cups in the egg membrane. However, once the fertilization membrane is raised, the perivitelline space exceeds the length of the nuclear filament, preventing other sperm from penetrating the egg. A fertilization cone forms around the nuclear filament of the penetrating sperm, but it does not appear to engulf the body of the sperm. Rather, the nuclear chromatin is injected into the egg as a long thread. The remaining sperm organelles are apparently abandoned on the egg surface. If this is the case, it would be a significant departure from fertilization in other molluscs and many other metazoans, in which sperm organelles, such as centrioles and mitochondria, enter the egg.

New sperm and egg characters, as well as significant differences in fertilization, indicate that Callochitonidae are basal to all other members of the order Chitonida and may warrant separation as the sister taxon to the suborders Chitonina and Acanthochitonina.

Received 7 December 1999; accepted 25 May 2000.

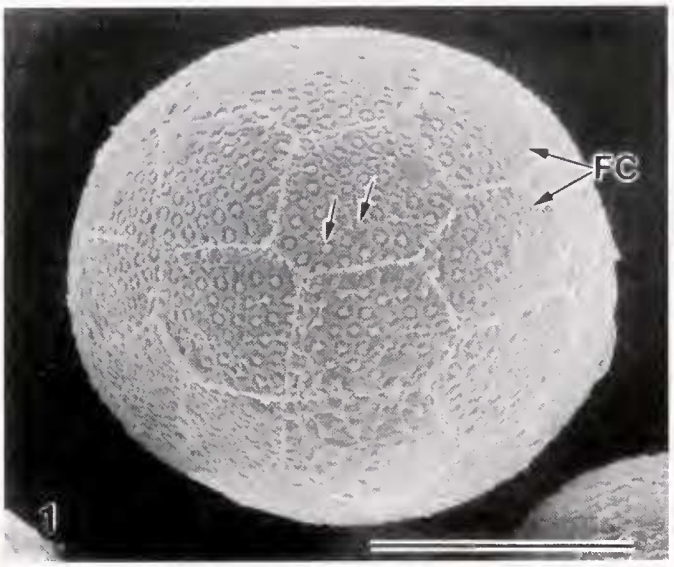
* To whom all correspondence should be addressed. E-mail: jbucklan@stfx.ca

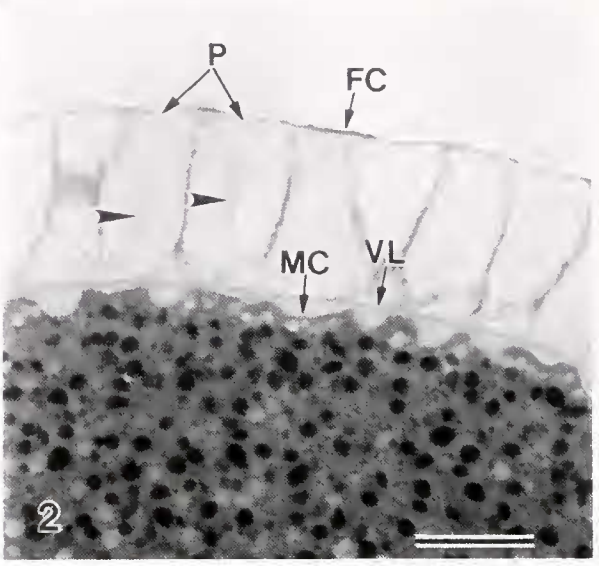
Introduction

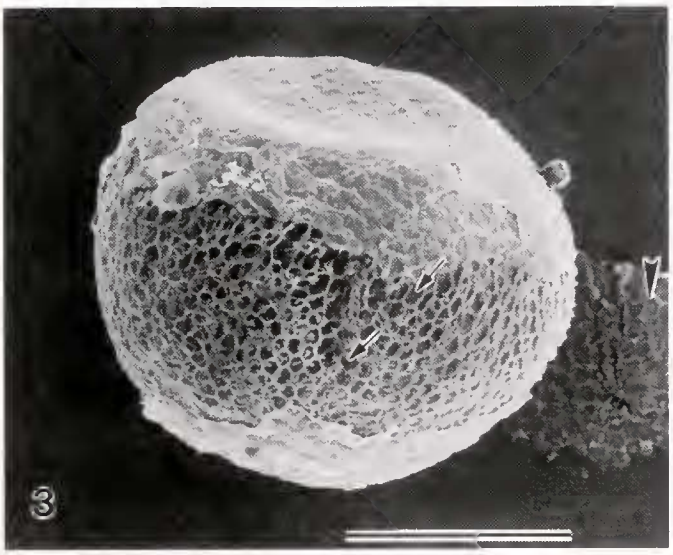
Sirenko (1997) recently classified extant chitons into two orders, Lepidopleurida (suborders: Lepidopleurina and Choriplacina) and Chitonida (suborders: Chitonina and Acanthochitonina). Lepidopleurida are considered basal to chitons in general, because they possess many primitive traits. For example, shell valve structure and articulation is simpler and ties in well with the fossil record (Sirenko, 1997); also they are the only chitons known to have typical aquasperm with prominent acrosomes, and eggs with smooth hulls (Hodgson *et al.*, 1988; 1989; Eernisse and Reynolds, 1994; Buckland-Nicks, 1995; Pashchenko and Drozdov, 1998). Presumably, in the ancestor of all members of Chitonida, the sperm acrosome became reduced to a minute vesicle atop a long needlelike extension of the nucleus, since this arrangement is found in all extant Chitonida (Buckland-Nicks *et al.*, 1990) but not in any Lepidopleurida examined to date (Hodgson *et al.*, 1988; Pashchenko and Drozdov, 1998). The intermediate condition of a prominent acrosome and short nuclear filament was recently discovered in the lepidopleurid *Deshayesiella curvata* (Pashchenko and Drozdov, 1998).

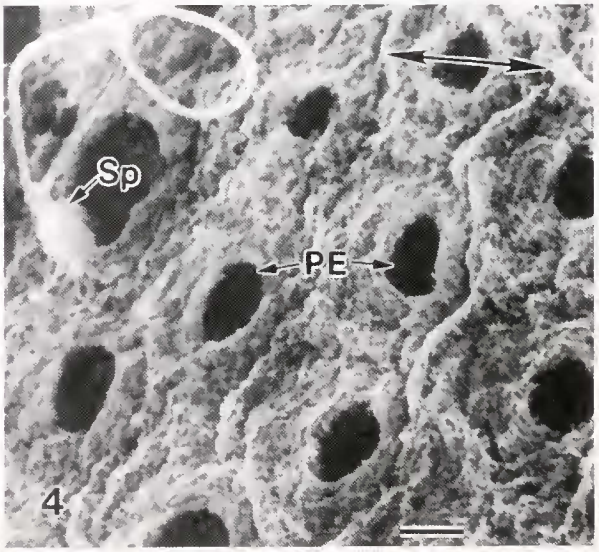
The term egg "hull" is used here instead of "chorion" as in previous publications (Buckland-Nicks *et al.*, 1988a, b; Buckland-Nicks, 1993, 1995), to describe the noncellular envelope enclosing the egg and its vitelline layer, because Richter (1986) showed that both the vitelline layer and the hull of chitons are formed by the egg and not by the follicle cells as in other Metazoa (for review of terminology, see Eernisse and Reynolds, 1994).

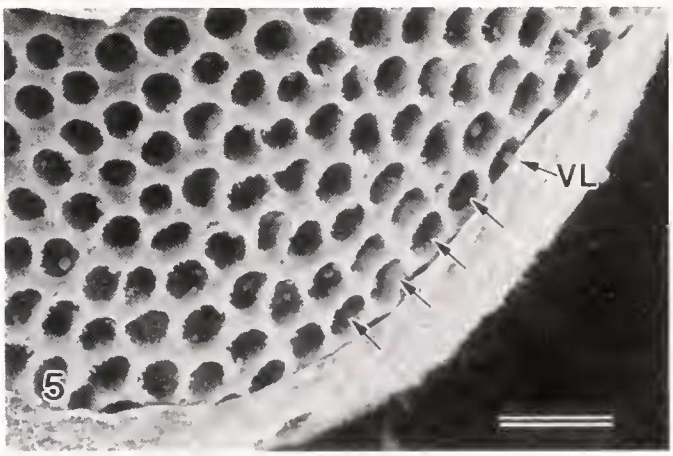
Chiton eggs evolved from having smooth unspecialized hulls, with fertilization presumably occurring anywhere on the surface, to having elaborate spinous or cupulous hulls that focus sperm to specific regions of the egg surface

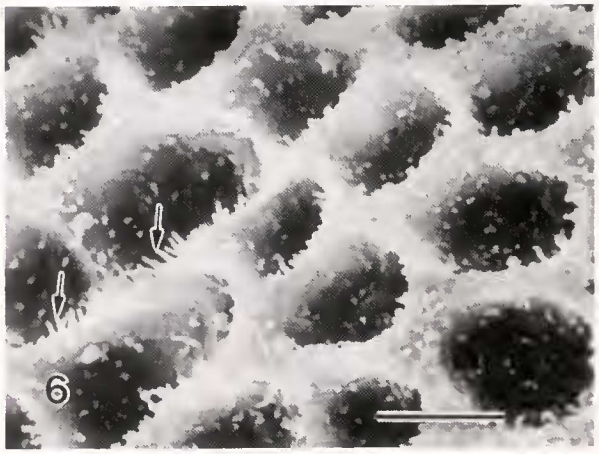

FC
1


P
FC
MC
VL
2


3


Sp
PE
4


VL
5


6

(Buckland-Nicks, 1993, 1995). Among Lepidopleurida, *Leptochiton asellus* has a smooth hull comprising a thick homogeneous jelly coat (Hodgson *et al.*, 1988), whereas *D. curvata* egg hulls are composed of a jelly coat that is penetrated by regularly spaced pores (Pashchenko and Drozdov, 1998). The mechanism of fertilization must be quite different in these lepidopleurids compared to Chitonina such as *Stenoplax conspicua* (Buckland-Nicks, 1995), or Acanthochitonina such as *Tonicella lineata* (Buckland-Nicks *et al.*, 1988b), because of basic differences in sperm acrosomes and egg hull structure. Sperm structure of different species has been found to be an accurate indicator of phylogenetic relationship and sometimes is used as grounds for reclassifying species to alternative taxa (Jamieson, 1987; Healy, 1988; Hodgson *et al.*, 1996).

The family Callochitonidae, although placed within the order Chitonina and regarded as "evolutionarily advanced" (Sirenko, 1997), exhibits several plesiomorphic traits (Buckland-Nicks, 1995) and is therefore one of several groups that are important to investigate in clarifying the phylogeny of chitons. This study describes in detail, for the first time in *Callochiton castaneus*, the structure of the egg hull and the mechanism of fertilization. The information gained is discussed in relation to the evolution of mechanisms of fertilization in chitons, as well as to chiton phylogeny in general.

Materials and Methods

Specimens of *Callochiton castaneus* Wood, 1815, were obtained in breeding condition from beneath intertidal rocks at East London (33° 03′ S; 28° 03′ E) and Port Alfred (33° 52′ S; 26° 53′ E), South Africa, from August to October 1999. The animals were brought back to the laboratory and placed individually in 60-mm petri dishes half-filled with 0.45-μm filtered seawater (FSW). Some individuals of each sex had spawned by the second day. Alternatively, eggs or sperm were obtained by removing the foot and digestive gland and puncturing the dorsal gonad.

If the specimen was male, the white "dry" sperm concentrate would ooze from the punctured testis and could be easily aspirated into a pipette. For a "concentrated sperm suspension," one drop of dry sperm was diluted in 5 ml of FSW. For a "dilute sperm suspension," one drop of the latter was further diluted in 5 ml of FSW. If the animal was female any free-spawned eggs were collected from the petri dish; otherwise the eggs were flushed from the ovary by aspirating a stream of FSW into the gonad. Eggs were then pipetted individually into another clean petri dish containing FSW, to reduce debris. Batches of about 50 eggs were removed into six small glass vials containing 4 ml of FSW. Two vials served as unfertilized controls. Two vials received three drops of diluted sperm suspension, and in two vials the FSW was replaced with concentrated sperm suspension. The eggs in one set of vials were transferred to primary fixative (see below) after 30 s, and the eggs in the second set of vials were fixed after 10 min.

Light microscopy

A few eggs were removed from control and experimental vials during the experiment to monitor the progress of fertilization and to be photographed using an Olympus BX50F-3 light microscope equipped with bright field and DIC optics. Slides were made by pipetting a few eggs onto a clean glass slide, placing pieces of coverslip around to make a well, then adding a whole coverslip, drying off excess water, and finally sealing the coverslip with nail varnish.

Sperm activity was noted to be highly variable. We tested the effects of serotonin (5-hydroxytryptamine) on sperm activity by mixing a drop of sperm concentrate with 1 ml of serotonin solution, to give a final concentration ranging from 1 to 10 μM serotonin. One drop of this sperm solution

Figures 1–6. Micrographs of unfertilized eggs of *Callochiton castaneus*.

Figure 1. Ripe egg with intact layer of follicle cells (FC) dissected from the ovary. Note regular arrangement of pores in hull (arrows) visible beneath follicle cells. Scale bar = 100 μm.

Figure 2. Light micrograph of 1-μm section of egg removed from the ovary, showing a layer of intact follicle cells (FC) and regularly spaced pores (P) in the jelly hull; the pores penetrate to the vitelline layer (VL) overlying the egg membrane cups (MC). Note meshwork of fibers (arrowheads) supporting pore structure. Scale bar = 10 μm.

Figure 3. Similar to Figure 1 except the egg has been rolled on sticky tape, which removed follicle cells and pore openings (arrowhead), thus revealing pores in the jelly hull (arrows). Scale bar = 100 μm.

Figure 4. Close-up of a spawned egg in which the follicle cells have retracted, revealing the arrangement of pores in naked jelly hull. Pore entrance (PE) is usually one-third of the diameter of the pore itself (double arrow), thus restricting sperm entry. A sperm (Sp) is visible at the entrance to one damaged pore. Scale bar = 2 μm.

Figure 5. Vitelline layer (VL) of the egg has been rolled off on sticky tape, revealing a regular series of membrane cups (arrows) that match up with pores in the hull. Scale bar = 15 μm.

Figure 6. Close-up of Figure 5, showing egg membrane cups with microvilli that are prominent on the raised edges (arrows) but sparse in the bases of cups. Scale bar = 5 μm.

was placed on a slide next to a separate drop of control sperm in filtered seawater, and observed periodically.

Electron microscopy

The primary fixative was made by mixing 1 ml of 25% glutaraldehyde with 9 ml FSW and adding this to 10 ml of 0.2 *M* Na cacodylate buffer (pH 7.4). This gave a final concentration of 2.5% glutaraldehyde in 0.1 *M* Na cacodylate buffer and FSW, to which was added 0.1 *M* sucrose. The fixative was refrigerated before use. Samples were fixed overnight and then washed in two changes of 0.1 *M* Na cacodylate buffer in FSW (pH 7.4) before post-fixing for 1 h in 1.5% osmium tetroxide in the same buffer. Fixed eggs were rinsed in distilled water and dehydrated in an ethanol series to 100%. At this stage about half of the eggs in each vial were removed to a second series of vials containing 100% ethanol for preparation for scanning electron microscopy (SEM).

In samples destined for transmission electron microscopy, ethanol was replaced with propylene oxide and then the eggs were transferred through infiltration media consisting of mixtures of propylene oxide and TAAB 812/Araldite CY212 resin (75:25, 50:50, 25:75) for 2 h in each mixture in capped vials (after Cross, 1989). Samples were exchanged into pure resin and left overnight in uncapped vials in a desiccator. The next afternoon, with the aid of a dissecting microscope, groups of five to six eggs were aspirated into BEEM capsules half filled with resin. Eggs were allowed to sink and then were arranged into the center of each mold using a stainless steel insect pin. Labels were added, and the BEEM capsules were placed in a 60°C oven for 40 h. Thick sections were cut with glass knives in an LKB 8800 ultratome, transferred to glass slides, and stained with 1% toluidine blue for about 20 s before rinsing with distilled water and air drying. Thin sections, with silver/gold interference color, were cut on a diamond knife (Diatome) and picked up on naked 150-mesh copper grids. Sections were reverse stained with aqueous lead citrate for 1 min, followed by aqueous uranyl acetate for 2 min, after the method of Daddow (1986). Stained sections were examined and photographed in a JEOL 1210 transmission electron microscope operated at 80 kV.

Samples destined for SEM were exchanged through an amyl acetate series to 100% and then aspirated under a dissecting microscope into Teflon flow-through specimen vials (Pelco) before capping and critical point drying. Subsequently, individual Teflon vials were uncapped and inverted on an SEM stub coated with a carbon sticky tab. The eggs stuck fast but could be rolled with an insect pin to remove the egg hull and expose either the vitelline layer or the egg membrane itself. Some eggs were cut in two with a Kesei microknife (Japan). Stubs prepared in this way were coated with gold in a Polaron E5100 sputter coater. Stubs were examined and photographed in a JEOL JSM 840 scanning electron microscope.

Results

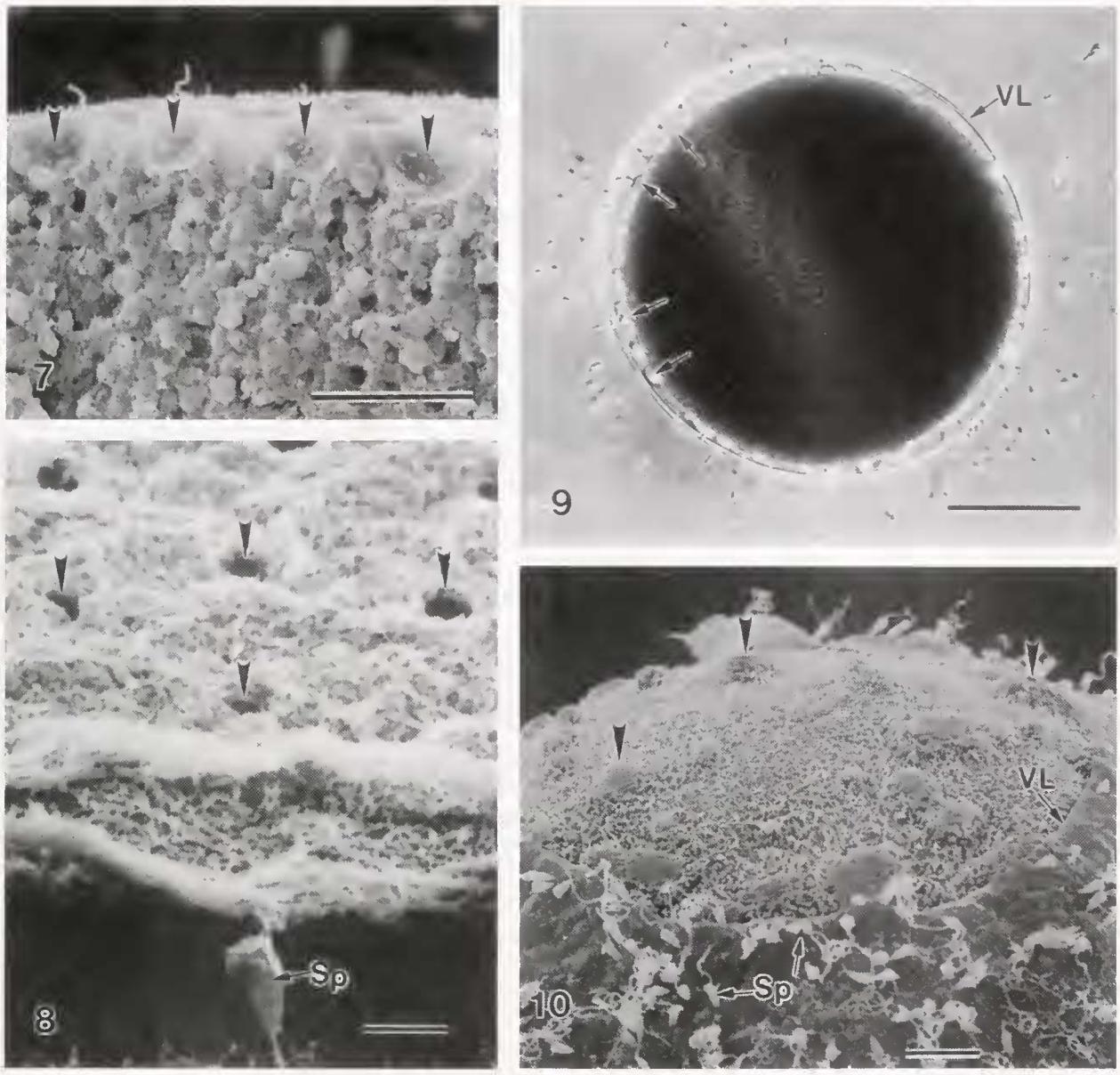
Morphology of the egg

The unspawned ripe eggs of *Callochiton castaneus* are about 220 μm in diameter and surrounded by a vitelline layer and a smooth, 20-μm-thick egg hull enclosed by a single layer of follicle cells, usually hexagonally disposed (Figs. 1, 2). The egg hull is made up of a delicate jelly coat supported by a fibrous matrix that is permeated by a series of pores spaced at regular intervals of 9 μm all over the surface (Figs. 3, 4). If unspawned eggs are rolled on sticky tape to remove the follicle cells, the regular array of pores in the jelly coat becomes visible (Fig. 3). At its entrance, each pore is about 2 μm in diameter (Fig. 4), but below this the diameter enlarges to about 8 μm (Fig. 2). Furthermore, there is a network of fibers that criss-cross each pore from apex to base (Fig. 2). Opposite, but below, the point at which the pores contact the vitelline layer, the egg membrane is formed into a series of cups (Figs. 5, 6). The depression in each cup usually coincides with a pore, and the lip of each cup coincides with the division between two pores (Fig. 2). The raised edges of the cups are rich in microvilli that penetrate into the vitelline layer (Figs. 2, 6); in the base of the cups, microvilli are sparse (Fig. 7).

The mechanism of fertilization

Soon after spawned eggs contact seawater, any remaining follicle cells retract, thus exposing the pores in the hull (Figs. 4, 8). In dilute sperm suspensions, sperm quickly locate the entrance to these pores and swim down to the egg surface (Fig. 8). If a concentrated sperm suspension is used, many sperm arrive at the egg surface simultaneously, overriding any potential block to polyspermy (Fig. 9). In these cases the jellylike hull is dissolved in 1 or 2 min, leaving the exposed vitelline layer of the egg coated with thousands of penetrating sperm, many of which induce fertilization cones (Figs. 9, 10). The fragile jelly coat degenerates in about an hour even under natural conditions, and it was not preserved intact by routine fixation.

When a fertilizing sperm penetrates the vitelline layer, the needlelike nuclear filament bridges the perivitelline space and egg cup to reach and fuse with the egg membrane (Figs. 11-13). The distance between the base of each egg cup and the vitelline layer varies from 1.5 –4 μm (Fig. 2), but the elongate nuclear filament permits sperm-egg fusion up to a distance of 6 μm (Fig. 12). Contact between sperm and egg results in their fusion and the formation of a narrow tube ($<$ 0.1 μm in diameter) through which the threadlike chromatin is injected into the egg cortex (Fig. 14). This is visible as a thin white thread when stained with Hoechst's 33358 DNA



Figures 7–10. Micrographs of fertilized eggs of *Callochiton castaneus*: SEM = scanning electron micrograph; DIC LM = differential interference contrast light micrograph.

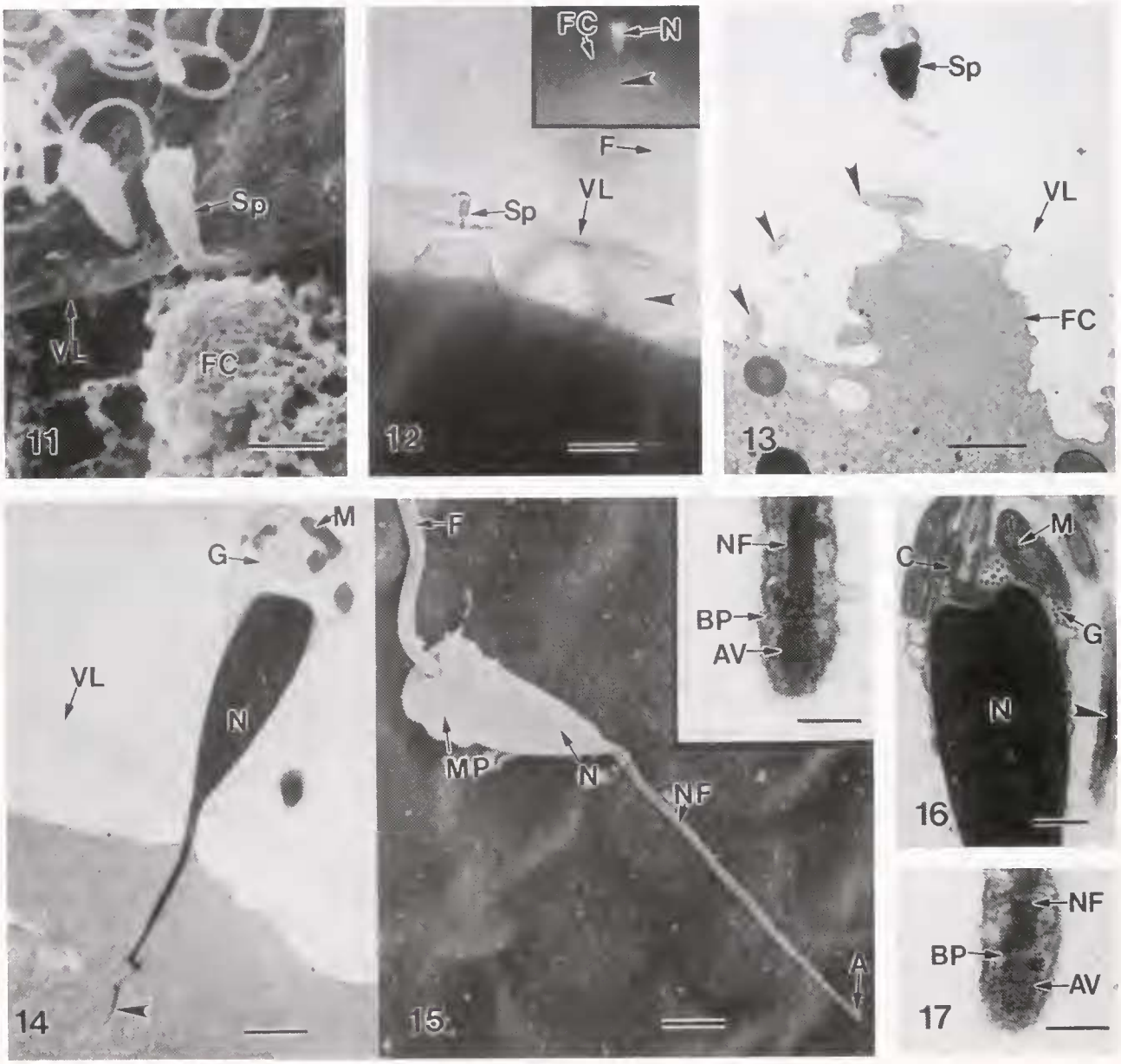
Figure 7. SEM of an unfertilized egg split in half with a Kesei microknife to show egg membrane cups (arrowheads). The vitelline layer has been removed. Scale bar = 10 μm.

Figure 8. SEM view of broken edge of the jelly hull, showing regular arrangement of pores above (arrowheads) and penetrating sperm (Sp) on vitelline layer below. Scale bar = 2 μm.

Figure 9. DIC LM of a polyspermic egg showing numerous fertilization cones (arrows) beneath the elevated vitelline layer (VL). Scale bar = 70 μm.

Figure 10. SEM of a polyspermic egg showing fertilization cones (arrowheads) beneath the vitelline layer, which has been partly removed by rolling the egg on sticky tape. Note also numerous sperm (Sp) on region of intact vitelline layer (VL). Scale bar = 10 μm.

stain and viewed under UV light (Fig. 12 inset). A fertilization cone is raised up around the penetrating nuclear filament from the surrounding egg cortex, as well as from fusion of adjacent egg microvilli (Fig. 13). However, the fertilization cone remains below the barrier of the vitelline layer and engulfs only the nuclear filament (Figs. 12, 14). The vitelline membrane raises up and forms the fertilization membrane (Figs. 9, 12). The raised edges of the egg membrane cups retract from the vitelline layer, creating a larger (6–7 μm) perivitelline space that excludes late-arriving



Figures 11–17. Micrographs of sperm and fertilized eggs of *Callochiton castaneus*: SEM = scanning electron micrograph; TEM = transmission electron micrograph; DIC LM = differential interference contrast light micrograph.

Figure 11. SEM of polyspermic egg that has been rolled on sticky tape, stripping the vitelline layer (VL) next to a penetrating sperm (Sp) and revealing the fertilization cone (FC) beneath it. Scale bar = 2 μm.

Figure 12. DIC LM of polyspermic egg showing one sperm (Sp) that has penetrated the vitelline layer (VL) and induced a fertilization cone, and a second, late-arriving sperm that cannot reach the egg membrane with its nuclear filament (arrowhead). Note also sperm flagellum (F) exhibiting large amplitude beat. **Inset:** Same as Figure 12 except that the penetrating sperm has been labeled with Hoechst's 33358 DNA stain and photographed under UV epifluorescence, revealing nucleus (N) injecting chromatin (arrowhead) into the egg cortex through the fertilization cone (FC). Scale bar = 6 μm.

Figure 13. TEM of a fertilization cone (FC) with part of a penetrating sperm (Sp) visible above the vitelline layer (VL). Note that the fertilization cone incorporates elevated cytoplasm as well as microvilli (arrowheads). Scale bar = 2 μm.

Figure 14. TEM of penetrating sperm injecting chromatin (arrowhead) into egg cortex. Note that the vitelline layer (VL) is intact except for a small pore through it. Sperm mitochondria (M) and remnants of glycogen (arrow) have collected in the membrane bag posterior to the nucleus (N). Compare with Figure16. Scale bar = 1 μm.

sperm (Fig. 12). The sperm organelles—including the centrioles, flagellum, and mitochondria, as well as some residual glycogen granules—collect in a bag of membranes above the fertilization membrane and do not appear to enter the egg cortex (Figs. 11, 14).

Morphology of the sperm

The sperm of *Callochiton castaneus* (described by Hodgson *et al.*, 1988) has a bullet-like nucleus extending into a long nuclear filament tipped by a minute acrosome, but it has retained a relatively unspecialized mid-piece (Figs. 15–17). Hodgson *et al.* (1988) believed that *C. castaneus* sperm did not possess an acrosome. Careful reexamination of their sections, however, revealed the presence of the minute acrosomal vesicle, which is separated from the nuclear extension by a basal plate (Figs. 15, 17). It was not possible to discern any subdivision of the acrosomal vesicle in this species. The main body of the nucleus is 3 μm long, and the nuclear filament is a further 6 μm. The mid-piece comprises five oblong mitochondria arranged fairly symmetrically around the centrioles (Figs. 15, 16).

Sperm dissected from some males were inactive even after being placed in seawater. However, all became active within 1 min following the addition of 1 μM serotonin. The degree of activity increased with increasing concentrations of serotonin, up to the maximum tested concentration of 10 μM.

Discussion

Morphology of the sperm

Except in the suborder Lepidopleurina, the acrosome of all chitons examined has been reduced to a small vesicle at the tip of a needlelike nuclear filament, which is an extension of the main body of the nucleus (see review by Buckland-Nicks, 1995). Partial reduction of the acrosome is noted among species of Lepidopleurina such as *Deshayesiella curvata* (Pashchenko and Drozdov, 1998). However, sperm of a number of families in this basal suborder have not been examined; these include Hanleyidae, Choriplacidae, and Nierstraszellidae. Acrosomes are fully reduced in Callochitonidae, although the mid-piece of sperm in this family has retained the primitive state, in which mitochondria are symmetrically disposed around the centrioles and there is no reinforcement of the flagellum (Buckland-Nicks, 1995). In most Chitonina the mitochondria are asymmetrically distributed around the centrioles and, in addition, the flagellum is reinforced near the annulus (Buckland-Nicks, 1995).

The activation of sperm by serotonin, which was observed here for *C. castaneus*, has not been previously recorded in chitons, although this response is well known from bivalves (Juneja *et al.*, 1993) and has also been noted in limpets (Buckland-Nicks and Howley, 1997; Buckland-Nicks and Hodgson, unpubl. data). In bivalves, serotonin has been shown to initiate sperm motility and egg maturation, as well as to improve fertilization success (Juneja *et al.*, 1993). This preliminary evidence for a function of serotonin in chiton reproduction brings hope that induction of spawning also may be possible. Until now, the inability to predict spawning has hampered studies of chiton reproduction.

Morphology of the egg

In Chitonida the egg hull is resilient, easily preserved, and elaborated into spines or cupules that not only slow the sinking rate but direct sperm to specific locations on the egg surface (Buckland-Nicks, 1993, 1995). The egg hulls of most Chitonina have elaborate spines with narrow bases and highly variable tips (Eernisse, 1984; Sirenko, 1993). Eernisse (1984) first suggested developing independent character sets based on hull spine structure and gill placement to test the validity of phylogenies based solely on shell valve morphology (Smith, 1960; Van Belle, 1983). Sirenko's subsequent investigations proved that these characters (1993), as well as variation in the articulamentum (1997) are useful in the analysis of chiton phylogeny. The new sperm and egg characters described here for *C. castaneus* will be important in future cladistic analyses of the Chitonina because they indicate that Callochitonidae are basal to Chitonina.

The egg hull of *C. castaneus* differs in some key respects from that of other chitons studied; in particular, it is unlike that of any other Chitonina. The vitelline layer is enclosed by a fragile, smooth jelly coat that is permeated by large pores. However, this type of hull may also occur in *Deshayesiella curvata* (Lepidopleurina); drawings of this species show a similar jelly coat containing regularly spaced pores (Pashchenko and Drozdov, 1998), although no micrographs of this feature have been published. In fertilization experiments with polyspermic eggs of *D. curvata*, it was

Figure 15. SEM of sperm, showing acrosome (A) at tip of nuclear filament (NF), main body of nucleus (N), mid-piece (MP), and flagellum (F). Scale bar = 1 μm. **Inset:** TEM of apex of sperm revealing acrosomal vesicle (AV) separated from nuclear filament (NF) by basal plate (BP). Scale bar = 0.7 μm.

Figure 16. TEM of sperm nucleus (N) and mid-piece showing mitochondria (M), centrioles (C), and glycogen granules (G). Note portion of nuclear filament (arrowhead). Scale bar = 0.7 μm.

Figure 17. TEM of apex of sperm revealing acrosomal vesicle (AV) separated from nuclear filament (NF) by basal plate (BP). Scale bar = 0.7 μm.

observed that the jelly coat disintegrated, much like that of *C. castaneus* (Buckland-Nicks and Sirenko, unpubl. results). This phenomenon of a fragile jelly coat is a plesiomorphy shared between Lepidopleurina and Callochitonidae, which excludes all other Chitonina studied thus far.

In species that have egg hulls with closed cupules, sperm penetrate the hull exclusively between the cupules, usually where their hexagonal bases meet (Buckland-Nicks, 1995). Eggs of some Acanthochitonina, such as *Lepidochitona dentiens* and *L. fernaldi*, have micropores in this region. These permit easier access to the vitelline layer, although sperm have two granules in the acrosome, suggesting that the hull may still represent a barrier (Buckland-Nicks *et al.*, 1990). *C. castaneus* appears to have a simpler acrosome structure, which would correlate with the provision of direct access to the vitelline layer by large pores in the jelly hull.

In summary, *C. castaneus* gametes and those of the Lepidopleurina share plesiomorphic characters such as a simple arrangement of mitochondria in the sperm mid-piece and a smooth egg hull. Yet *C. castaneus* shares the derived characters of nuclear extension and reduction of acrosome with all other Chitonida. A previous cladistic analysis of chitons, which was largely based on sperm and egg characters, predicted that Callochitonina was distinct from Chitonina (Buckland-Nicks, 1995). The present study corroborates this prediction and indicates that *C. castaneus* is unique and perhaps should be placed in a sister taxon to both Chitonina and Acanthochitonina.

The mechanism of fertilization in chitons

Fertilization in *C. castaneus* and other Chitonida bears some similarity to that in other molluscs, in the sense that a sperm acrosome releases enzymes that digest a pore in the egg envelope, enabling the inner acrosomal membrane to fuse with the egg membrane (Buckland-Nicks *et al.*, 1988, this study). However, in many other respects the mechanism of fertilization in these chitons is highly derived when compared with those of other molluscs and of metazoans in general.

Firstly, there is no extrusion of an acrosomal process or "perforatorium" by the polymerization of actin, as occurs throughout molluscs and other metazoan groups (see review by Tilney, 1985). Rather, in all members of Chitonida the permanent needlelike nuclear filament has replaced the perforatorium (Buckland-Nicks *et al.*, 1988b, 1990). The intermediate condition of a short nuclear extension found in *Deshayesiella curvata* (Pashchenko and Drozdov, 1998) suggests that reduction in acrosome size among lepidopleurids may be linked with an increase in the length of the nuclear filament. Furthermore, all lepidopleurids examined have a subacrosomal granule, which in other metazoans is composed of actin for extruding the perforatorium during fertilization.

A second important difference observed between Chitonida and other molluscs is that the sperm organelles, as well as most of the nuclear membrane, apparently remain on the surface of the egg (Buckland-Nicks *et al.*, 1988b; Buckland-Nicks, 1995; this study). No chiton sperm has been observed becoming completely engulfed by a fertilization cone. The probable reason for this is that the vitelline layer, disturbed only by a minute pore permitting penetration of the nuclear filament, remains a barrier to the envelopment of the sperm by the fertilization cone. In other molluscs such as bivalves, as well as in many other metazoans, the vitelline layer is breached and the fertilization cone raises up through it to engulf the entire sperm, including part of the flagellum (see reviews by Tilney, 1985; Longo, 1987). In these species there is an initial paternal contribution of centrioles and mitochondria to the egg at fertilization, although, with the exception of some bivalve molluscs (Hoeh *et al.*, 1991), the paternal mitochondria degenerate and do not contribute to the zygote. Furthermore, in sea urchins and some other metazoans, a sperm centriole contributes to the movement of the pronuclei as well as to the formation of the mitotic spindle prior to first cleavage (see review by Gilbert, 1999). Chitons may be unique among molluscs if, in addition to the exclusion of paternal mitochondria, the centrioles that form the mitotic spindle are also maternally derived. Confirmation of this derivation will require appropriate labeling of sperm centrioles and mitochondria before and after fertilization.

To better understand how the mechanism of fertilization has evolved in chitons, it will be important to examine fertilization in a species like *Leptochiton asellus*, which has a typical molluscan acrosome (Hodgson *et al.*, 1988) and perhaps a mechanism of sperm entry more similar to that of limpets or bivalves.

Acknowledgments

We thank Robin Cross and Shirley Pinchuk for assistance in setting up electron microscopes and ancillary equipment, as well as Marvin Randall for doing most of the darkroom work. We are grateful to Doug Eernisse for important and helpful criticisms. This research was supported by a Hugh Kelly Fellowship from Rhodes University and an NSERC of Canada research grant, both to J.B-N., as well as a Rhodes University research grant to A.N.H.

Literature Cited

Buckland-Nicks, J. 1993. Hull cupules of chiton eggs: parachute structures and sperm focusing devices? *Biol. Bull.* **184:** 269–276.

Buckland-Nicks, J. 1995. Ultrastructure of sperm and sperm-egg interaction in Aculifera: implications for molluscan phylogeny. Pp. 129–153 in *Advances in Spermatozoal Phylogeny and Taxonomy*, B. G. M. Jamieson, J. Ausio, and J.-L. Justine, eds. Mem. Mus. Natl. Hist. Nat. **166**, Paris.

Buckland-Nicks, J., and B. Howley. 1997. Spermiogenesis and sperm structure in relation to early events of fertilization in the limpet *Tectura testudinalis* (Muller, 1776). *Biol. Bull.* **193:** 306–319.

Buckland-Nicks, J., R. Koss, and F.-S. Chia. 1988a. The elusive acrosome of chiton sperm. *Int. J. Invertebr. Reprod. Dev.* **13:** 193–198.

Buckland-Nicks, J., R. Koss, and F.-S. Chia. 1988b. Fertilization in a chiton: acrosome-mediated sperm-egg fusion. *Gamete Res.* **21:** 199–212.

Buckland-Nicks, J., F.-S. Chia, and R. Koss. 1990. Spermiogenesis in Polyplacophora, with special reference to acrosome formation. *Zoomorphology* **109:** 179–188.

Cross, R. H. M. 1989. A reliable epoxy resin mixture and its application in routine biological transmission electron microscopy. *Micron Microsc. Acta* **20:** 1–7.

Daddow, L. Y. M. 1986. An abbreviated method of double lead stain technique. *J. Submicrosc. Cytol.* **18:** 363–372.

Eernisse, D. G. 1984. *Lepidochitona* Gray, 1821 (Mollusca: Polyplacophora), from the Pacific coast of the United States: systematics and reproduction. Ph. D. Thesis, University of California at Santa Cruz.

Eernisse, D. 1988. Reproductive patterns in six species of *Lepidochitona* (Mollusca: Polyplacophora) from the Pacific Coast of North America. *Biol. Bull.* **174:** 287–302.

Eernisse, D. J., and P. D. Reynolds. 1994. Polyplacophora. Pp. 55–110 in *Microscopic Anatomy of Invertebrates, vol 5: Mollusca 1.* Wiley-Liss, New York.

Gilbert S. F. 1999. *Developmental Biology*, 6th ed. Sinauer, Sunderland, MA. 749 pp.

Healy, J. M. 1988. Sperm morphology and its systematic importance in the Gastropoda. *Malacol. Rev. Suppl.* **4:** 251–266.

Hodgson, A. N., J. M. Baxter, M. G. Sturrock, and R. T. F. Bernard. 1988. Comparative spermatology of 11 species of Polyplacophora (Mollusca) from the suborders Lepidopleurina, Chitonina and Acanthochitonina. *Proc. R. Soc. Lond. B* **235:** 161–177.

Hodgson, A. N., S. Ridgeway, G. M. Branch, and S. J. Hawkins. 1996. Spermatozoal morphology of 19 species of prosobranch limpets (Patellogastropoda) with a discussion of patellid relationships. *Proc. R. Soc. Lond. B.* **351:** 339–347.

Hoeh, W. R., K. H. Blakely, and W. M. Brown. 1991. Heteroplasmy suggests limited biparental inheritance of *Mytilus* mitochondrial DNA. *Science* **251:** 1488–1490.

Jamieson, B. G. M. 1987. A biological classification of sperm types, with reference to annelids and molluscs, and an example of spermiocladistics. Pp. 311–332 in *New Horizons in Sperm Cell Research*, H. Mohri, ed. Japan Science Society, Tokyo.

Juneja, R., S. J. Segal, and S. S. Koide. 1993. Promotion of fertilizability of *Spisula* oocytes with 5-hydroxy-tryptamine. *Invertebr. Reprod. Dev.* **24:** 103–106.

Longo, F. J. 1987. *Fertilization.* Chapman and Hall, New York.

Pashchenko, S. V., and A. L. Drozdov. 1998. Morphology of gametes in five species of far-eastern chitons. *Invertebr. Reprod. Dev.* **33:** 47–56.

Richter, H. -P. 1986. Ultrastructure of follicular epithelia in the ovary of *Lepidochitona cinerea* L. (Mollusca: Polyplacophora). *Dev. Growth Differ.* **28:** 7–16.

Sirenko, B. I. 1993. Revision of the system of the order Chitonida (Mollusca: Polyplacophora) on the basis of correlation between the type of gills arrangement and the shape of the chorion processes. *Ruthenica* **3:** 93–117.

Sirenko, B. I. 1997. The importance of the development of articulamentum for taxonomy of chitons (Mollusca, Polyplacophora). *Ruthenica* **7:** 1–24.

Smith, A. G. 1960. Amphineura. Pp. 141–176 in *Treatise on Invertebrate Paleontology, Part I, Mollusca 1*, R. C. Moore, ed. Geological Society of America, New York.

Tilney, L. G. 1985. The acrosomal reaction. Pp. 157–213 in *The Biology of Fertilization, Vol. 2*, C. Metz and A. Monroy, eds. Academic Press, New York.

Van Belle, R. A. 1983. The systematic classification of chitons (Mollusca: Polyplacophora). *Informations de la Société Belge de Malacologie. Série* **11:** 1–164.